

## ABSTRACT

Title: PRELIMINARY GENETIC  
CHARACTERIZATION OF RIBOSOMAL  
PROTEIN L10 IN *SACCHAROMYCES*  
*CEREVISIAE*  
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While recent research has focused on the exciting concept of the ribosome as a ribozyme, ribosomal proteins have been largely overlooked. This study focuses on four previously identified mutants of the ribosomal large subunit protein L10, in an effort to better understand how the protein's structure corresponds to its function. All of the mutants studied displayed how the embedded nature of L10 caused only slight conformational changes to impact function of the ribosome as a whole; in these cases primarily altering the interaction between the ribosomal A-site and aminoacyl-tRNAs.

GENETIC CHARACTERIZATION OF RIBOSOMAL PROTEIN L10 IN  
*SACCHAROMYCES CEREVISIAE*

By

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## Dedication

To Anni, John, and most especially Matthew,  
without whom this body of work would not exist

## Acknowledgments

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## Chapter 1: Introduction

All organisms employ large ribonucleoprotein complexes known as ribosomes in order to catalyze the process of protein synthesis. From our earliest understanding as an RNA scaffolding unit solely extant for the positioning of the proteins responsible for performing the process of translation, to the more recent understanding of the ribosome as a ribozyme [1,2], the ribosome's central role in the function of the cell has made it a subject of intense scrutiny. Recent research, however, has focused on the centrality of the rRNA to the functions of the ribosome, instead of the ribosomal proteins [3].

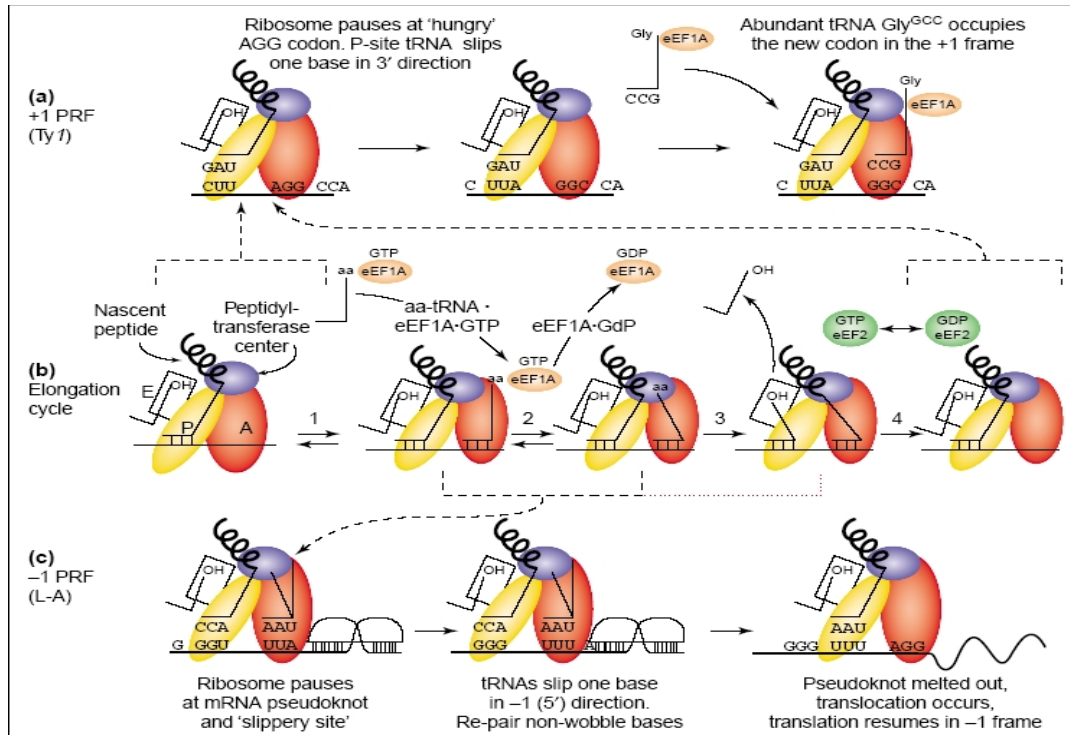
### *Maintenance of Frame by the Translating Ribosome*

Since the role of the ribosome is primarily to translate mRNA into polypeptides, maintenance of translational reading frame is a crucial aspect of its function. Despite this stricture, a surprising number of elements work to undermine this function. Slipping, hopping, and shunting all allow the ribosome to deviate from normal decoding rules [4,5]. Programmed ribosomal frameshifting (PRF) is the most widespread variant of such mechanisms; it is found in animal, plant, and fungal viruses, bacteriophages, and even some cellular genes [6-8]. PRF is a non-random response to *cis*- acting signals within mRNA transcripts that cause translating ribosomes to shift reading frame, usually by a single nucleotide in the 5' (-1) or 3' (+1) direction, allowing them to continue elongation in the new frame (Figure 1;

[4,6,7,9-11]). These *cis*-acting signals and their corresponding effects on PRF are well characterized; *trans*-acting factors less well so. Many viruses of clinical, veterinary, and agricultural importance use PRF as a method of maximizing coding space in a space-limiting genome [12-14]. Additionally, PRF can serve as a regulatory mechanism to control the relative amounts of proteins produced. For example, the L-A virus of yeast employs a -1 PRF to produce its Gag-pol fusion protein [15,16], and if this delicate balance is disturbed by a change in PRF efficiency, virus maintenance is placed in jeopardy [reviewed in 17]. Such a change in -1 PRF efficiency in this case results also in the loss of the killer phenotype due to the loss of L-A 's M<sub>1</sub> satellite virus, which encodes a killer toxin [reviewed in 18]. Similarly, TyI, a yeast retrotransposable element, uses +1 PRF to produce its Gag-pol protein, a process which evidences a similar sensitivity to changes in frameshifting efficiencies as its -1 PRF counterpart. Remarkably, the basic molecular mechanisms of -1 PRF is identical in yeast and humans [6,17].

#### *Ribosomal Proteins and Maintenance of Frame*

This serendipitous relationship between ribosome function and viral maintenance can be manipulated to examine ribosomal component function [reviewed in 7]. PRF efficiency can be affected by various structural modifications within the ribosome that lead to alterations of normal ribosome function. Previous findings have linked changes in +1 PRF to changes in the translocation step of the translation elongation pathway [19] and -1 PRF to the accomodation step (Figure 1; [11]). The natures of two large subunit proteins, for example, have previously



**Figure 1:** The 'integrated model' of programmed ribosomal frameshifting

In Ty 1-mediated +1 PRF (a), the presence of the AGG in the 0-frame of the heptameric CUU AGG C 'slippery site' (where the incoming frame is indicated by spaces) causes elongating ribosomes to pause while awaiting delivery of the rare CCU-tRNA<sup>Arg</sup>. Slippage of the ribosome by one base in the 3' direction during this pause allows the P-site tRNA to base-pair to the +1-frame GGC codon. The new A-site codon corresponds to the highly abundant CCG-tRNA<sup>Gly</sup>. When viewed in the context of the elongation cycle, this slip can only occur when the A-site is empty; that is, after translocation and before insertion of aa-tRNA in complex with eukaryotic Elongation Factor 1A (eEF1A) into the A-site. The elongation cycle (b) can be divided into four general steps: (1) selection of cognate aminoacyl-tRNA (aa-tRNA); (2) accommodation of the 3' end of the aa-tRNA into the ribosomal A-site; (3) peptidyl transfer; and (4) eukaryotic Elongation Factor 2 (eEF2) mediated translocation. In a -1 PRF signal (c) an mRNA pseudoknot causes elongating ribosomes to pause with their A- and P-site tRNAs positioned over the heptameric X XXY YYZ 'slippery site' (where the incoming frame is indicated by spaces). The G GGU UUA slippery site from the L-A dsRNA virus of yeast is shown here. While paused at the slippery site, if the ribosome shifts by one base in the 5' direction, the non-wobble bases of both the A- and P-site tRNAs can re-pair with the new -1-frame codons. As both A- and P-sites must be occupied by tRNAs, in theory this shift should occur after step 1 but before step 4 of the elongation cycle (indicated by black and red dotted lines). The experimental data suggest that the -1 shift is actually limited to pre-peptidyltransfer ribosomes (black dotted lines). Taken from [11]; used with permission.

been elucidated by our lab in this fashion. Cells with the *mak8-1* allele of *RPL3* were found to be unable to maintain the killer phenotype [20,21], and were later found to have elevated -1 PRF efficiencies [22]. Ribosomes isolated from cells with *mak8* alleles showed decreased peptidyltransferase activities [23], reinforcing the idea that post-accomodation ribosomes with both A and P sites occupied pause for longer periods of time at -1 frameshift signals, thus increasing -1 PRF efficiency [24]. The residues involved in the mutants studied are highly conserved, and located near the peptidyltransferase center [25]. Mutants of *RPL5*, on the other hand, caused increased frameshifting efficiencies in both the -1 and the +1 directions. Further analyses of these mutants using pharmacological methods found that sparsomycin, a known P-site associated peptidyltransferase inhibitor [26], was antagonistic to the frameshifting defects, which points to L5's role in anchoring peptidyl tRNA to the P site [27].

### *RPL10*

In yeast, L10, a 25.4 kDa ribosomal large subunit protein, is encoded by the single-copy essential gene *RPL10*. It has also been previously identified as *GRC5* (Growth Control; [28]), *QSRI* (Quinol-cytochrome c reductase Subunit Requiring; [29]), and *RIX7* (Ribosomal eXport; [30]) . Its expression has been linked to the suppression of tumors in a Wilm's tumor cell line [31], and is it is known to be essential for subunit joining [32] and for 60s subunit stability [33]. It has no exact counterpart in *E.coli*, but in archaea, plants, and animals there are homologues (Figure 2; [31,34,35]). L10 is located on the lateral stalk of the large subunit, and makes contact with almost all of the functional regions of the large subunit: 5S rRNA,

the A-minor motif in helix 38, helix 39, the GTPase-associated center, and the P-loop (Figure 3; [25]). Given its location, one would expect mutations of this strategically placed protein to yield information about how general structure of ribosomal proteins may influence function, and, specifically, whether L10 is functionally compartmentalized or whether it is responsible for coordinating information from the many regions of the ribosome it contacts, as well as indicate with more precision the roles of L10 within the ribosome.

#### *RPL10 mutants*

With this goal in mind, our lab obtained a variety of *RPL10* alleles from the lab of Dr. Arlen Johnson (Table 1). These alleles had been found, in unrelated screens, to express various mutant phenotypes. *ts942* was found by the Jacobsen lab via a temperature-sensitivity screen for factors involved in mRNA turnover – a process in which translation plays an important part. This point mutation was found to decrease the decay rates of unstable mRNAs, even though overall protein synthesis was not decreased [35].

Microscopic study and FACs analysis of *grc5-1<sup>ts</sup>* indicated a role for L10 in cell morphology and in growth regulation [37], and this mutant displayed a half-mer phenotype in polysome profiles, due to the disruption of intersubunit binding [38,32]. Intriguingly, a nearby double-amino acid substitution, *qsr1-24*, displayed a considerably different phenotype than its neighbor; it was found to have only a slight temperature dependent loss of viability and did not show the previous phenotype's related accumulation of cell wall material [39]. A fourth mutation, *qsr1-1*, has been

```

CLUSTAL W (1.82) multiple sequence alignment

gi|57284207 REKRVRPFPVS YRGRQTHFHS AWSYRYCKNKPYPKSRFCRGVPDAKIRIF 50
YLR075W    ---MARRP-----ARCYRYQKNKPYPKSRYNRAVPDSKIRIY 34
gi|55231756 ---MSDKP-----ASMYRDI DKPAYTRREYITGIPGSKIAQH 34
              *          *  **  .: .: .: .: .: .: .: .:

gi|57284207 DLGRKKAKVDEFPLCGHMVSDEYEQLSSEALEAARICANKYMKVSCGKDG 100
YLR075W    DLGKKKATVDEFPLCVHLVSNELEQLSSEALEAARICANKYMTTVSGRDA 84
gi|55231756 KMGRKQKDADDYPVQISLIVEETVQLRHGSLEASRLSANRHLIKELGEEG 84
.: : : : .: : : : .: : *  **  : : : : : : : : .: .: .:

gi|57284207 -FHIRVRLHFPFHVIRINKMLSCAGADRLQTGMRGAFGKPPQGTIVARVHIGQ 149
YLR075W    -FHLRVRVHFPFHVLRINKMLSCAGADRLQQGMRGANGKPHGLAARVDIGQ 133
gi|55231756 -YKMTLRKFPHQVLRNKKQATGAGADRVSDGMRAAFGKIVGTAAARVQAGE 134
[: : : * .: .: : *  **  : : : : : : : : .: .: .:

gi|57284207 VIMSIRTKLQNKHEHVIEALRRAKFKFPGRQKIHSKKWGF TKFNADEFED 199
YLR075W    IIFSVRTKDSNKKDVVVEGLRRARYKFFGQQKIILSKKWGF TNLDRPEYLK 183
gi|55231756 QLFTAYCINVEDAEHVKEAFRRAYNKITPSCKIKVERGEELLIA----- 177
: : : : .: : *  **  : : : : : : : : : : : : :

gi|57284207 MVAEKRLIPDGGGVKYIPNRGPLD-----KWRALHS 230
YLR075W    KREAGEVKDDGAFVKFLSKKGSLENNIREFPPEYFAAQA 221
gi|55231756 -----

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Figure 2: *RPL10* Homology

ClustalW alignment [36] of human, yeast, and *H. marismortui* *RPL10* sequences.

Boxes enclose mutation sites addressed in this study.

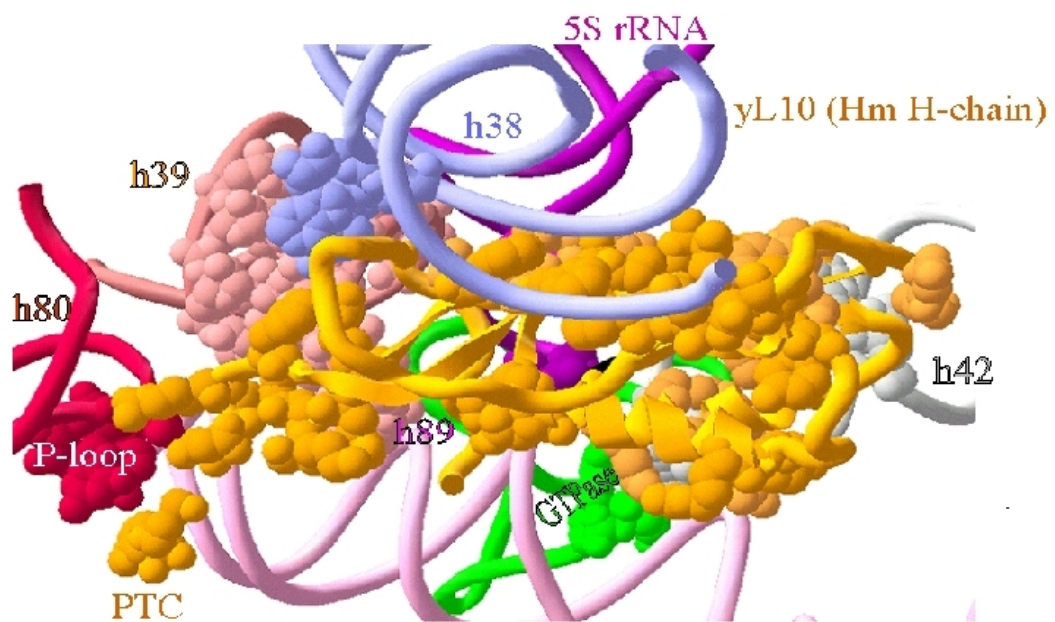


Figure 3: L10 and environment

L10 is located between many major players in the translating ribosomes. Pictured here is L10e (“H-chain”) and nearby large subunit rRNA, color-coded according to region.

strain	mutation	genotype	references
<i>ts942</i>	F85S	MATa lys2Δ0 met15Δ0 leu2Δ0 ura3Δ0 his3Δ0	Zuk et al 99
<i>grc5-1<sup>ts</sup></i>	G161A	MATa leu2Δ0 ura3Δ0 <i>grc5-1<sup>ts</sup></i>	Koller et al 96
<i>qsr1-24</i>	KI164-5WM	MATα ade2Δ1, ura3Δ1, trp1Δ1, can1Δ100 <i>qsr1</i> Δ1::His3 + pDEQ2[CEN LEU2 <i>qsr1</i> -24]	Eisinger et al 97
<i>qsr1-1</i>	G194A	MATα ade2Δ1, his3Δ11,15, ura3Δ1, leu2Δ3,112, trp1Δ1, can1Δ100, <i>qsr1</i> Δ1	Tron et al 95
JD1243	wild type	MATa met15Δ0 leu2Δ0 ura3Δ0 his3Δ0; <i>rpl10</i> ::Kan covered by RPL10 URA3 2μ vector	Zuk et al 99
JD758	-	MATa <b>kar1Δ1</b> arg1 L-A HN M1	Tumer et al 98
JD759	-	MATα <b>kar1Δ1</b> arg1 thr(i,x) L-A HN M1	Tumer et al 98

**Table 1: Yeast Strains**



shown to display extremely slow growth due to markedly diminished rates of protein synthesis [33], and its polysome profile was remarkably similar to that of *qsr1-24*. Given the phenotypes of these mutations, it seems likely that further information regarding L10 could be gleaned from them by applying the methods and tools so successfully used on other ribosomal proteins in our lab.

### Summary

L10 is a multifunctional protein whose wide range of function is heavily influenced by its structure. It seems likely, therefore, that further investigation into this intriguing structure/function relationship is likely to yield rich information about L10, the ribosome, and the process of translation. I pursued the following: a strictly genetic method, focusing on the relationship of ribosome structure to maintenance of frame and frameshifting during translation, and a pharmacogenetic analysis to investigate the effects of certain antibiotics on mutant forms of *RPL10*.

## Chapter 2: Materials and Methods

### Strains, General Procedures, and Media

The *S. cerevisiae* strains used in this study were a generous gift from Dr. Arlen Jacobsen and are listed in Table 1. Due to their temperature sensitivities, mutant strains were grown at 25°C unless otherwise noted. Mating types were confirmed as previously described [40]. Confirmation of auxotrophic markers was done via replica plating on media lacking the appropriate nutrient. Plates of YPAD, YPG, SD, synthetic complete media, and 4.7MB were prepared as described previously [41]. DNA sequence analysis was performed by the Center for Biosystems Research DNA Sequencing Facility. *Escherichia coli* strain DH5alpha was used to amplify plasmids as needed, and both transformations using this strain and yeast transformations were carried out as described previously [42]. Plasmids were purified from *E. coli* using a Qiagen kit, and from yeast by phenol extraction of cellular lysates. Plasmids used in this study are listed in Table 2.

### Killer Assay

Cytoduction of the L-A and M<sub>1</sub> viruses into rho<sup>0</sup> strains was done as previously described [41] and cells were picked that were SD- and YPG+. These cells were then scored for their ability to maintain the killer phenotype.

The killer virus assay was carried out as previously described [16]. Yeast colonies were replica plated onto 4.7MB plates freshly seeded with 0.5 O.D.<sub>595</sub> of the

<b>plasmid</b>	<b>function</b>	<b>source</b>
pJD419	<i>LEU2</i> based zero frame reporter	J.D. Dinman
pJD420	<i>LEU2</i> based -1 frame reporter	J.D. Dinman
pJD421	<i>LEU2</i> based +1 frame reporter	J.D. Dinman
pJD633	<i>TRP1</i> based zero frame reporter	J.D. Dinman
pJD634	<i>TRP1</i> based -1 frame reporter	J.D. Dinman
pJD635	<i>TRP1</i> based +1 frame reporter	J.D. Dinman

**Table 2: Plasmids**

killer indicator strain 5 x 47. Killer activity was defined as a zone of growth inhibition surrounding K<sup>+</sup> colonies after 3 days at 20°C.

#### dsRNA visualization

dsRNA of L-A and M<sub>1</sub> viruses was visualized using native agarose-TAE gel electrophoresis and ethidium bromide staining after total nucleic acid extraction performed as described previously [43].

#### Dual Luciferase Assay

Dual luciferase assays were performed as previously described [44] using lysates of cells harboring the appropriate 0-frame, -1 frame, or +1 frame plasmids (Table 2). Reactions were carried out using the Dual-Luciferase Reporter Assay System from Promega Corporation; readings were obtained using a Turner Designs TD20/20 luminometer. The firefly/*Renilla* activity ratio generated from the zero-frame reporter was divided into that from the frameshift reporters and multiplied by 100% to obtain frameshift efficiencies for each recoding signal. All assays were performed in triplicate at least three times. Other statistics were performed as described in [45].

### Dilution Spot Assay

Drug sensitivity assays were carried out as described previously [46]. Ten-fold serial dilutions of equal numbers of cells grown to saturation were spotted onto medium containing sparsomycin (5 µg/ml), anisomycin (5 µg/ml), or paromomycin (600 µg/ml), as well as YPAD. Sensitivity was indicated by either a severe reduction or a complete lack of growth.

### Computational Analyses

The crystal structure of the *H. marismortui* ribosome [25] was visualized using the Swiss PDB viewer.

## Chapter 3: Results

### Frameshifting

The killer phenotypes of the *rpl10* mutants and that of the wild type strain are shown in Figure 4A. *qsr1-24* is clearly K<sup>-</sup>. Analysis of the dsRNA indicates that *qsr1-24* appears to be M<sub>1</sub><sup>-</sup> (Figure 4B), suggesting that the loss of the killer phenotype is the inability of *qsr1-24* to support propagation of M<sub>1</sub>.

Changes in -1 PRF can cause loss of the killer phenotype in yeast. Therefore, as a quantitative measure of frameshifting, dual luciferase assays were performed to determine the exact percentage of PRF (Figure 5). The double mutant *qsr1-24* exhibited the most obvious deviation from wild type, with an efficiency of -1 PRF increased by more than two-fold of the wild type rate. A similar degree of variation in +1 PRF by this mutant was not found, which suggests the defect is more likely to be found in association with the ribosome's A-site. Both *ts942* and *grc5-1<sup>ts</sup>* exhibit smaller increases in their -1 frameshifting efficiencies, whereas *qsr1-1* mimics wild type most closely. Little deviation was seen from wild type levels in +1 frameshifting, with all strains frameshifting near wild type efficiency.

### Pharmacogenetics

In order to test the effects of the mutant forms of L10 on ribosomal affinity for aminoacyl- and peptidyl-tRNAs, a pharmacogenetic approach was carried out. (Figure 6) Anisomycin is known to bind in the A-site of the peptidyltransferase center [26], thereby inhibiting the binding of the acceptor end of the aminoacyl-tRNA into

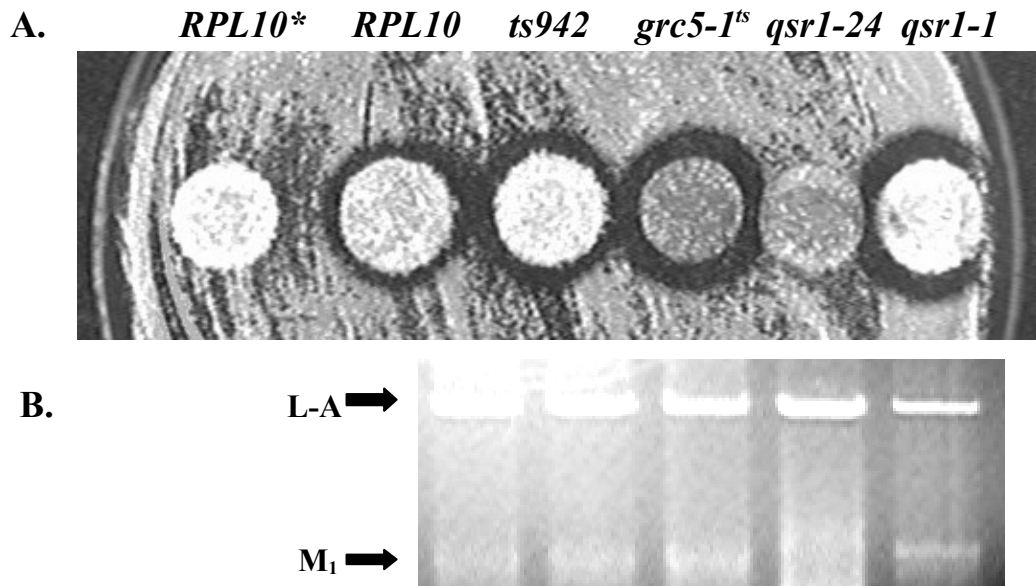


Figure 4: The mutants of RPL10 have differing effects on the yeast killer virus.

(A) Killer assay of strains harboring either wild type or mutant alleles of *RPL10*.

Killer activity is observed as a zone of growth inhibition around the colonies. A wild-type strain not inoculated with the killer virus (*RPL10\**) is included for comparison.

(B) Total RNA extracted from the strains described in (A) were separated through a 1% TAE agarose gel. L-A and M<sub>1</sub> form bands at 2.5 and 1.8 kbp respectively, as indicated.

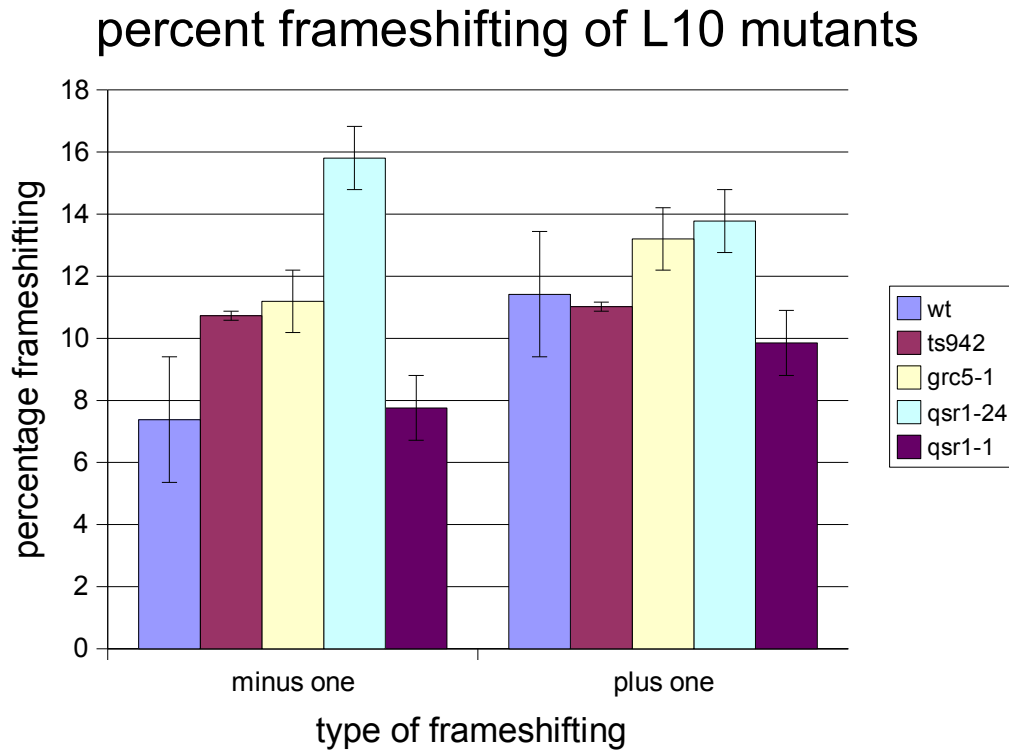


Figure 5: Efficiencies of -1 and +1 frameshifting in wild type and mutant strains. Efficiencies are depicted as percent frameshifting, which was calculated by dividing firefly/Renilla luminescence data from the appropriate reporter by similar ratios generated from cells expressing a zero-frame control plasmid.



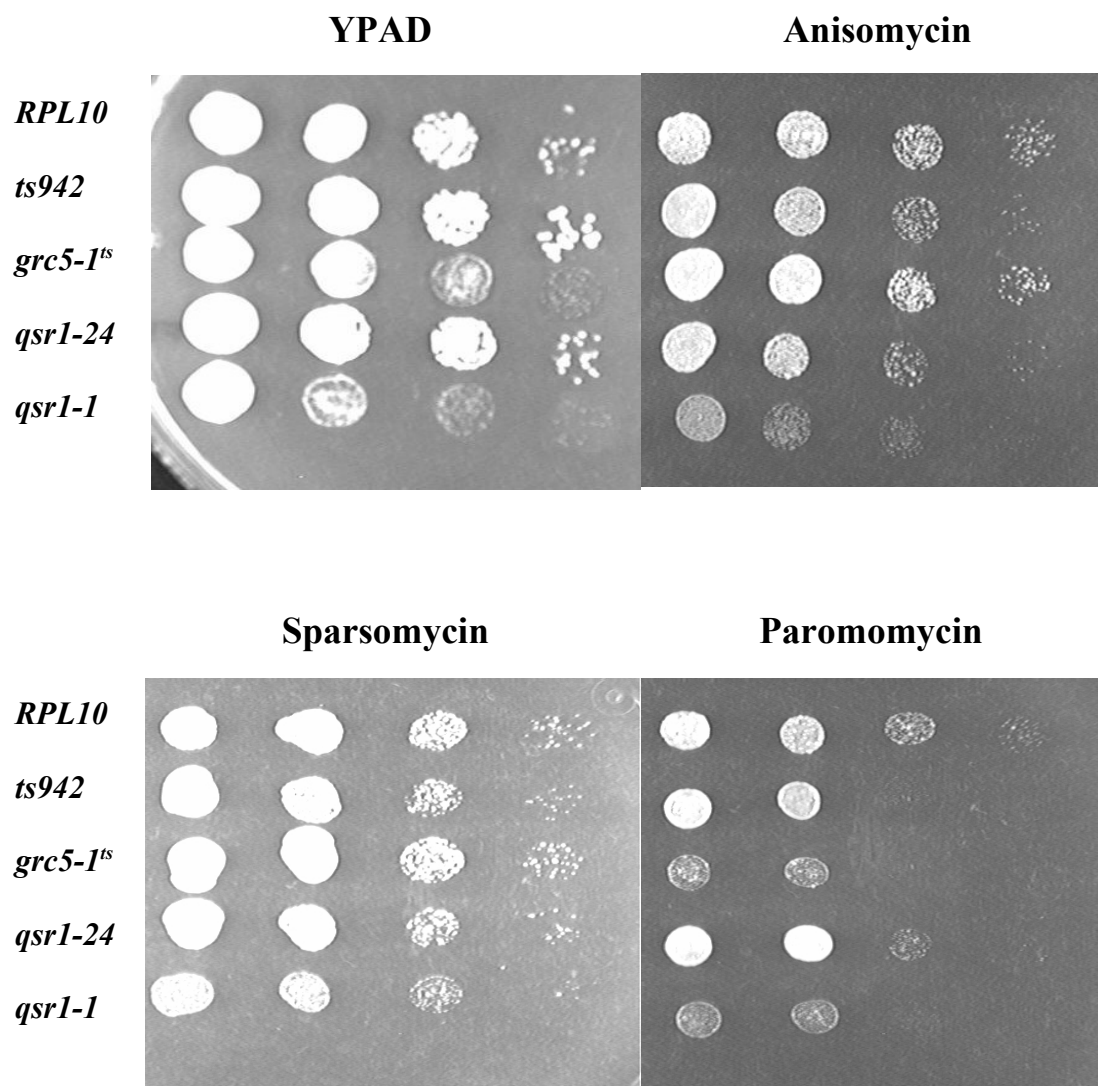


Figure 6: Strains harboring mutant forms of L10 have altered drug sensitivities.

Mid log phase cultures of the strains were spotted in ten-fold dilutions onto plain and drug-containing (anisomycin, 5µg/ml; sparsomycin, 5µg/ml; puromomycin, 600µg/ml) media and grown for three days at 25°C.

peptidyltransferase center [47,48], and thus is a good indicator of changes specific to the A-site. On anisomycin containing media, *grc5-I<sup>ts</sup>* exhibits a wild-type phenotype, whereas *ts942* and *qsr1-24* are slightly affected, and *qsr1-1* displays the most altered phenotype. The exacerbated effect of anisomycin on these three mutants suggests a higher affinity of ribosomes containing these forms of L10 for aa-tRNAs, and point to these mutations, particularly that of *qsr1-1*, as A-site specific. When assayed for growth in the presence of sparsomycin, an indicator of P-site specific changes [reviewed in 49], little deviation from the wild-type profile was seen. Paromomycin, another A-site specific probe known to stabilize the binding of near cognate tRNAs [50-52], caused hypersensitivity relative to wild type in *grc5-I<sup>ts</sup>*, which was least affected by anisomycin, reflecting the slight difference in binding between anisomycin and paromomycin [50,26]. *ts942* and *qsr1-24* also showed increased sensitivity to paromomycin, providing further confirmation of their A-site specificity.

## Chapter 4: Discussion

Without proper maintenance of frame by the ribosome, cellular life could not exist. This, as well as the many other crucial functions of the ribosome, depend on the protein-protein and protein-RNA interactions within the structure. As such, ribosomal proteins have come to the forefront of recent research on the relationship between ribosomal structure and function.

It has previously been shown that L10 plays a crucial role in cellular and ribosomal function, impacting everything from cell wall formation to nonsense-mediated decay and ribosomal export. Its precise role in the ribosome, however, is still under investigation. In this study, we have linked certain aspects of ribosomal function to specific sites within L10, particularly the mutation site in the strain *qsrI-24*.

### *qsrI-24*

Though located near *grc5-1<sup>ts</sup>*, the rare non-lethal double amino acid mutant at position 164-5 has a decidedly different phenotype from that of its neighbor. The mutation changes what was a polar, basic site to a bulky, nonpolar one at the site of the protein's closest approach to h42 – a site known for its flexibility during ribosomal translocation - and sandwiched between h42 and h38. The sharp increase in rates of -1 frameshifting abolished this strain's ability to support M<sub>1</sub> and its secreted toxin. Due to the embedded location of this mutation, it is perhaps unsurprising that most of the tested antibiotics had little effect on the strain relative to wild type. Since paromomycin's interaction with the ribosome occurs at the decoding center, the

observed paromomycin sensitivity offers one possible explanation for the observed changes in frameshifting. Perhaps this mutation points to a site involved in accuracy of the codon:anticodon contacts; the mutation alters this interaction sufficiently cause a more “slippery” A-site, leading to the observed alteration in in -1 PRF due to the intrinsically higher rate of error at the A-site.

All mutants examined in the study showed effects related to the A-site of the ribosome, as expected, due to the location of L10 right where aa-tRNAs are loaded onto the large subunit. *qsr1-24*, in particular, merits further study to determine the precise role the KI<sub>164-5</sub> site plays in A-site function. Furthermore, the strains used in this study were non-isogenic, and subsequent studies should be conducted to investigate whether the observed deviant phenotypes remain in isogenic strains.

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